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Imaging light-modulated release of synaptic vesicles in the intact retina: Retinal physiology at the dawn of the post-electrode era

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Abstract

Here, we illustrate an optical method for directly measuring the light-regulated synaptic output of neurons in the retina. The method allows simultaneous recording from many retinal neurons in intact flat-mount preparations of the vertebrate retina. These recordings depend on the use of FM1-43, an activity-dependent fluorescent dye that selectively labels synaptic vesicles. Release of the dye, which occurs upon vesicle exocytosis, is detected with 2-photon microscopy. This utilizes an infrared laser to trigger fluorescence excitation of the dye, while minimally perturbing retinal activity by activating phototransduction in rods and cones. Using this approach, one can measure activity of single neurons in the intact retinal network and populations of neurons in different layers of the retina, providing a new way to examine the function of retinal synapses and how visual information is processed.
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Keywords: FM1-43; Optical imaging; Ribbon synapse; Retina; Transmitter release; Light response

1. Introduction

Over the past 40 years, electrophysiology has been the main experimental tool for understanding how neurons in the retina respond to light and communicate with one another to process visual information. These recordings have elucidated the basic neural circuit of the retina and have explained the biophysical mechanisms underlying the initial phototransduction event in rods and cones and the subsequent synaptic events in downstream retinal neurons. Microelectrode and patch electrode recordings have provided a temporally accurate glimpse of the individual activities of retinal neurons in response to light. However, recent advances in optical recording methods provide new opportunities for functional studies of the retina, with several potential advantages. Optical recordings are relatively non-invasive, and depending on the type of indicator dye, can reveal different aspects of neuronal activity, including changes in intracellular ion concentrations, changes in membrane potential, and release of synaptic vesicles. Unlike single cell electrical measurements, optical recordings can be made from many neurons at once, revealing the 'big picture' of how the retina responds to light.

Our approach to functional imaging of the retina has utilized the fluorescent dye FM1-43 as an activity-dependent marker of vesicle exocytosis and endocytosis. FM1-43 is a lipophilic, styryl dye that increases in fluorescence when it partitions into biological membranes, thereby labeling the surface of cells (Cochilla, Angleson, & Betz, 1999; Ryan, 2001). Since it is amphipathic, FM1-43 inserts into the outer leaflet of the plasma membrane and cannot 'flip' into the inner leaflet. However, if a cell exposed to FM1-43 happens to be endocytosing membrane from the cell surface, the dye will become trapped inside internalized vesicles. By washing away the surface dye,

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one can selectively see and measure the label in these vesicles and thereby measure the rate of endocytosis. Subsequent exocytosis of labeled vesicles will allow the dye to be released from the terminal. Thus, by measuring the decrease in FM1-43 fluorescence from pre-loaded synaptic terminals, the rate of exocytosis can also be estimated.

FM1-43 and related dyes have been used for many years to study synaptic vesicle cycling in presynaptic terminals at the frog neuromuscular junction (Betz & Bewick, 1992; Betz, Mao, & Smith, 1996) and in synapses formed between dissociated hippocampal neurons in culture (Ryan, 2001). However, FM1-43 has been used very little to study synapses in more complex neural circuits (but see, Kay et al., 1999). At least in part, this results from technical difficulties. When added to freshly-obtained slices of the brain or retina, FM1-43 stains not only the presynaptic terminals, but other structures, including cell bodies and dendrites (Rea et al., 2004). This non-specific labeling obscures the synaptic labeling, decreasing the dye's usefulness as a selective marker of synaptic release. For reasons that are not completely explained, making retinal slices or dissociating individual retinal neurons dramatically alters the dye uptake pattern. We have discovered that simply by carrying out the loading procedure in intact retina, we can obtain synapse-specific labeling (Rea et al., 2004). Subsequent imaging of presynaptic terminals in dissociated cells, retinal slices, or retinal flat-mounts has provided new opportunities for studying how physiological stimuli, including light, and regulate synaptic vesicle release.

The key optical tool that we use to measure light-regulated FM1-43 release is 2-photon microscopy. FM1-43 release can be visualized with conventional one-photon microscopy, using visible light (488 nm) to excite the FM1-43 and produce a fluorescence signal. However, illumination with visible light will trigger phototransduction in retinal photoreceptors and elicit synaptic responses in downstream neurons, thereby perturbing the very process that we want to study. Two-photon microscopy utilizes intense pulses of infrared light. When used judiciously, this can excite FM1-43 fluorescence, while minimally triggering phototransduction in the photoreceptors (Choi et al., in press). Hence, 2-photon microscopy is like a '2-way mirror,' allowing the investigator to observe synaptic activity without the retina 'seeing' that it is being monitored. An additional advantage of 2-photon microscopy is the ability to obtain images at deep planes of focus through the entire thickness of the retina (~200 μm), because infrared light penetrates far into tissue.

Here, we combine the use of FM1-43 and 2-photon microscopy to reveal how light regulates exocytosis at two synapses in the retina. First, by imaging the outer plexiform layer (OPL) in retinas from various species, we show that release from terminals of cone photoreceptors is influenced by exposure to white light. Second, by imaging the inner plexiform layer (IPL) of retina from goldfish, we can detect how illumination affects vesicle release from ON bipolar cells, which have giant (~10 μm diameter) synaptic terminals. Isolated ON-bipolar cells from goldfish have become a classic system for studying the subcellular function of ribbon synapses (von Gersdorff & Matthews, 1999). The combination of FM1-43 and 2-photon microscopy now enables study of how this synapse responds to light in the intact system, putting the wealth of biophysical information obtained previously into physiological context.

2. Methods

2.1. Retinal preparation and dye loading

All procedures were approved by the UC Berkeley Animal Care and Use Committee. All animals were maintained on a 12:12 light:dark cycle.

Eyes were obtained from the lizard *Anolis segrei*. After 1 h of dark adaptation, the eye was hemisected and the retina was removed in the dark, with the retinal pigment epithelium (RPE) attached. The retina was cut into thirds and each piece was mounted RPE-side down, onto filter paper. Retinal flat-mounts were bathed in normal lizard saline containing 30 μM FM1-43 from 45 min to 1.5 h, followed by a 5 min wash with 1 mM ADVASEP-7, as described previously (Rea et al., 2004). All stages of retinal preparation were carried out at 21 °C in complete darkness, with the aid of an infrared converter. After the dye loading, the retinal flat-mounts were transferred to dye-free normal lizard saline and kept in darkness, or exposed to white light of intensity 10^7 photons/μm^2/s for 20–40 min before imaging. For slice preparation, FM1-43-loaded flat-mounts were transferred to Ca^{2+}-free lizard saline, and using a tissue slicer, cut into ~300 μm thick slices for imaging.

Eyes were obtained from the common goldfish *Carassius auratus* of 4–5 in. in length. Dissection was carried out in darkness as above for the lizard. Retinal flat-mounts were labeled with FM1-43 in goldfish saline in darkness, or in room light for 45 min to 1 h. After dye loading, the retinas were transferred to Ca^{2+}-free saline for imaging.

Eyes from larval tiger salamander *Ambystoma tigrium*, gecko *Gekko gecko*, and mouse (1-month-old) were obtained after dark adapting the animals overnight before the day of the experiment. Dissection and FM1-43 labeling of these tissues were carried out in darkness as above for the lizard. After dye loading, the retinas were transferred to Ca^{2+}-free saline for imaging.

2.2. Two-photon imaging and image analysis

The retina was imaged with a commercially available Zeiss 510 confocal system equipped with a Maitai

(Spectra Physics, Mountain View, CA) mode-locked Ti:sapphire laser (860 nm) and a 40× achroplan, 0.8 NA water-immersion objective. Images were acquired using Zeiss LSM software and analyzed using Scion Image software (Scion, Frederick, MD). Xara X software (UK) was used for image processing. For each image, a square region of retinal flat-mount (230×230 μm) was scanned. For 3-D reconstructions to produce the 'virtual slice' views, 600–700 optical sections focused 300 nm apart through the retina were obtained, starting from the vitreous side of the retina. Reconstructions of the FM-loaded retina were made using Imaris software (Bitplane, Zurich, Switzerland). For analysis of FM1-43 fluorescence of the goldfish ON bipolar cell terminals, an intensity profile across the diameter of the terminal (region indicated with a box in Figs. 5C and D) was generated for each terminal using Scion Image software. Intensity values from 50 to 70 terminals (four experiments for the dark condition, five experiments for the light condition) were averaged. Variability among data is expressed as means ± SEM.

3. Results

3.1. Loading FM1-43 in retinal synaptic terminals

We used physiological stimuli (darkness) to load synaptic vesicles in anole retina with FM1-43 dye. Fig. 1 illustrates our FM1-43 loading protocol for synaptic vesicles in the retinal whole-mount preparation, using the photoreceptor ribbon synapse as an example (Fig. 1A). When FM1-43 is added to the external solution, it labels the plasma membrane (Fig. 1B). As the tissue is kept in darkness, the photoreceptors tonically exocytose glutamate-containing synaptic vesicles and the dye becomes incorporated into recycled synaptic vesicles via compensatory endocytosis (Fig. 1C; Rea et al., 2004). Next, the labeled tissue is rinsed with ADVASEP-7 to remove surface membrane staining (Fig. 1D). ADVASEP-7 is a cyclodextrin with a hydrophobic core that rapidly binds FM1-43 with higher affinity than the lipid membrane (Kay et al., 1999). Incubating the dye-stained tissue with ADVASEP-7 dramatically reduces labeling of surface membranes, leaving the endocytosed synaptic vesicles as the only dye-labeled structures. Once the dye loading process is complete, we can present stimuli (light or dark) to the retina. When a bright light stimulus is presented, transmitter release is suppressed in photoreceptors and most of the FM1-43 fluorescence should remain in the photoreceptor synaptic terminal (Fig. 1E). In contrast, vesicle release continues in darkness and FM1-43 should be gradually lost from the terminal (Fig. 1F).

3.2. Visualizing FM1-43 in the intact retina

Two-photon microscopy is ideal for imaging the light-regulated release of FM1-43. Focusing through the thickness of the retina, we can image optical *z*-sections through an FM1-43-loaded retinal flat-mount to reveal the anatomy and activity of populations of neurons in each layer (Fig. 2). Since the anole lizard retina lacks rods, the only synaptic terminals in the OPL that release synaptic vesicles are the terminals of cone photoreceptors. Bipolar cell terminals are postsynaptic, receiving information from the photoreceptors, and horizontal cell feedback to cone terminals involves a non-vesicular mechanism (Fahrenfort, Klooster, Sjoerdsma, & Kamermans, 2005; Wu, 1992). Hence,

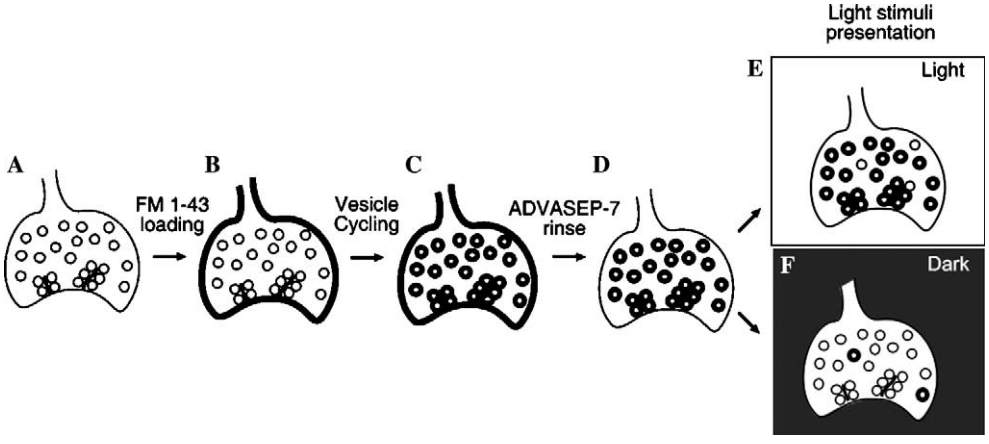

Fig. 1. Loading FM1-43 in retinal synaptic terminals. (A–D) FM1-43 dye loading into the retinal ribbon synapse. (A) The ribbon synapse is filled with synaptic vesicles and contains ribbons near active zones where the vesicles are released. (B) FM1-43 is lipophilic and partitions into the plasma membrane. (C) When the synapse is allowed to undergo endo- and exo-cytosis, for example, as photoreceptors are kept in darkness, FM1-43-labeled membrane is endocytosed and labeled synaptic vesicles fill the terminal. (D) ADVASEP-7 is used to wash FM1-43 from the external membrane, leaving only the internalized synaptic vesicles labeled. (E and F) FM1-43 dye unloading from the ribbon synapse. (E) When bright light stimulus is presented to FM1-43-loaded photoreceptor, vesicle release is suppressed and most of the labeled vesicles remain in the terminal. (F) In darkness, photoreceptor synapse continuously releases vesicles, and the dye is released from labeled vesicles as they undergo exocytosis.

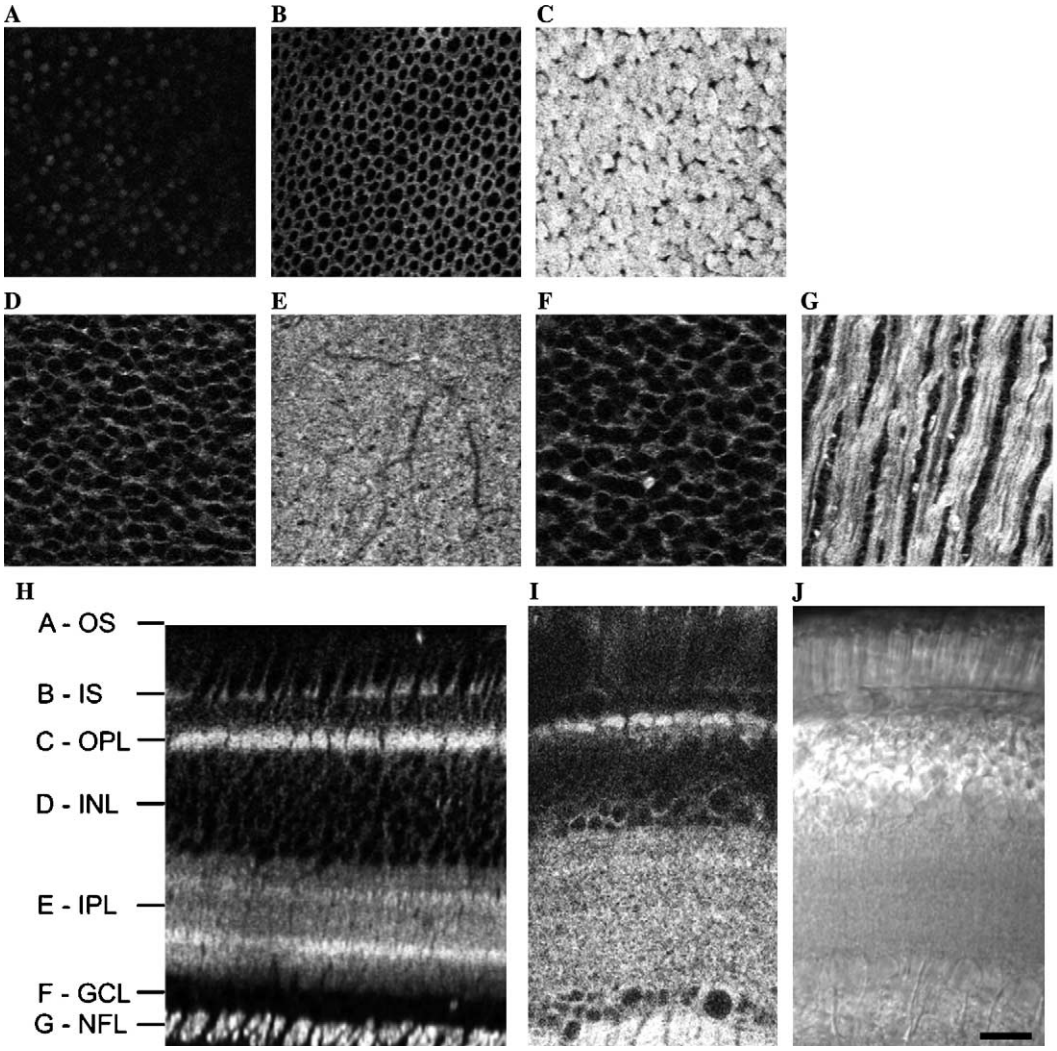

Fig. 2. Imaging FM1-43-labeled retinal flat-mount using 2-photon microscopy (A–G) Tangential optical sections through the lizard retina labeled with FM1-43 in the dark. (A) Cone outer segments (OS). (B) Cone inner segments (IS). (C) Cone synaptic terminals in the outer plexiform layer (OPL). (D) Inner nuclear layer (INL). (E) Inner plexiform layer (IPL). (F) Ganglion cell layer (GCL). (G) Nerve fiber layer (NFL). (H) A virtual slice created from reconstruction of ~600 optical *z*-sections focused 300 nm apart in depth. Approximate depths of sections shown in (A)–(G) are indicated with lines. (I and J) Retinal slice preparation. (I) FM1-43-labeled retinal flat-mount was cut into 300 μm thick slices and then imaged in the plane of the slice, to illustrate that same level of details of FM1-43 labeling can be imaged either by taking a series of tangential *z*-sections from a flat mount preparation or by making a slice preparation for imaging. (J) Brightfield image of the slice shown in (I). Scale bar is 20 μm in all panels.

FM1-43 specifically labels the large (4–8 μm in diameter) cone terminals in the OPL (Fig. 2C). Thus, FM1-43 labeling of the lizard OPL provides a unique opportunity for imaging activity of a two-dimensional array of a homogeneous population of neurons with single synapse resolution.

The IPL, which contains synaptic contacts between bipolar, amacrine, and ganglion cells, also exhibited bright labeling (Fig. 2E). In contrast, the non-synaptic layers, including outer- and inner- segments of photoreceptors (OS, IS), inner nuclear layers (INL), and ganglion cell layer (GCL) showed little labeling (Figs. 2A, B, D, and F). The labeling observed around IS may be attributed to the end feet of Muller glial cells, though the rest of the Muller cells excludes the FM1-43 dye and appear dark (Fig. 2E). FM1-43 strongly labeled the nerve fiber layer (NFL), which contains the axons of retinal ganglion cells (Fig. 2G). The mechanism of FM1-43 staining of these axons is unknown, but the axotomy that necessarily occurs during retinal isolation appears to result in dye uptake, similar to the uptake that we have observed when loading is carried out in retinal slices or acutely dissociated retinal neurons (Rea et al., 2004). Since 2-photon imaging results in very thin optical sections (<1 μm), the NFL labeling did not obscure or interfere with imaging of the deeper synaptic layers that are many micrometers from the NFL.

We have used 2-photon imaging to provide tangential views of the retina, but we are also able to assemble a computer reconstruction of a 'virtual slice' view through

the FM1-43-labeled retina by stacking together the individual *z*-sections. A reconstruction made with 600 optical *z*-sections focused 300 nm apart in depth is shown in Fig. 2H. The detail of the synaptic activity profile of the retina is strikingly similar to images taken of an actual retinal slice preparation (Figs. 2I and J).

3.3. Light-regulated FM1-43 release from cone terminals

We utilized the FM1-43-loaded lizard retina to monitor how light influences vesicle release from retinal neurons. Photoreceptors are depolarized in the dark and continually release transmitter, so the dye should unload spontaneously in the dark. Indeed, compared to fluorescence in the OPL imaged just after dye loading (Figs. 3A and C), we find that all cone synaptic terminals lost the dye after 40 min in the dark (Figs. 3B and D), reflecting tonic synaptic activity. The reconstructed 'virtual slice' view shows the dye loss was specific to the synaptic layers (compare Figs. 3C and D). The OPL showed dramatic dye loss from cone terminals. The IPL also showed some dye loss in the OFF sub-lamina, which contains synaptic terminals of OFF-bipolar cells and amacrine cells. The Off-bipolar cells should be depolarized and therefore, tonically releasing synaptic vesicles in the dark, which would account for the release of FM1-43. When the FM1-43-loaded retina was exposed to bright light, the fluorescence in the OPL barely decreased, and indicating nearly complete suppression of release from the cone synapse (Choi et al., in press; Figs. 3E and F). The labeling in the NFL showed little loss of fluorescence either in the light or the dark, consistent with the lack of synaptic vesicles in ganglion cell axons and therefore a non-vesicular location of the dye. Hence, we often used NFL fluorescence to normalize the fluorescence of the other layers, in quantitative determinations of release.

3.4. FM1-43 can be used in a wide variety of intact retinas

The combination of the use of activity-dependent synaptic dyes and 2-photon microscopy can be applied to various vertebrate retinas, including those from amphibians, reptiles, fish, and mammals. Salamander retina has been widely used for electrophysiological experiments for decades (Werblin, 1991). The OPL of the salamander shows a distinct pattern of FM1-43-labeled synaptic terminals of rod and cone photoreceptors (Fig. 4A). The duplex retina of salamanders provides an opportunity for comparing the different properties of synaptic release from rods and cones. The cone synapse is large and irregular in shape (arrow) and the rod terminal is smaller and round in shape (arrowhead). In contrast, anole lizards have an all-cone retina. We have taken advantage of this to quantify light-dependent synaptic transmission from the cones (Choi et al., in press; Fig. 2C). On the other hand, the retina of the gecko lacks cones and contains only rods, whose large terminals in the OPL also can be stained with FM1-43 (Fig. 4B). Together, the lizard and the gecko retina present another opportunity to study the responses of rods and cones independent of their interactions with one another.

FM1-43 can be used to examine synaptic function in photoreceptors from other vertebrate retinas, including goldfish (Fig. 4C), and mammals (mouse; Fig. 4D). Both the photoreceptors and their synapses tend to be small in mouse retina (<2 μm in diameter for the rod terminals shown in Fig. 4D). The small size of these synapses makes them difficult for electrophysiological studies, but should not preclude optical measurement of dye release. Optical imaging with high spatial resolution allows distinction not only between different mouse rod terminals, but also between subcellular compartments within the synaptic terminal, as illustrated by FM1-43 labeling that is bright around the periphery of the terminal and dim in the center.

These various retinas also showed FM1-43 labeling in the IPL. Labeling patterns were similar, with many small synapses throughout the layer incorporating the dye. In most of the IPL, the dense packing of terminals precluded identification and visualization of individual types of synapses, with certain exceptions. First, processes belonging to Muller cells spanned the depth of the IPL and excluded the dye, resulting in dark profiles (arrows in Figs. 4E–H). Second, in the goldfish retina, the IPL contained very large terminals (arrowhead in Fig. 4G) that showed differential FM1-43 loading in the light vs dark (see below). These terminals were from Mb type ON bipolar cells, and they could be distinguished from the Muller cell processes by their larger size and selective location in the ON-sub-lamina of the IPL (closest to the ganglion cell layer). The exclusion of the dye from these terminals in the dark is consistent with these cells being depolarized (ON) in the light and hyperpolarized in darkness.

3.5. Light-regulated FM1-43 release from ON-bipolar cell terminals

Photoreceptors transmit light information to ON and OFF-bipolar cells. The dendrites of ON bipolar cells in the OPL respond to glutamate that is tonically-released from photoreceptors in the dark, generating a hyperpolarization. The hyperpolarization generated in the OPL closes voltage-gated Ca^{2+} channels at the other end of the bipolar cell, in the IPL, thereby suppressing synaptic vesicle release from the presynaptic terminal. In response to light, ON bipolar cells depolarize, increasing the release of vesicles onto postsynaptic targets, including retinal ganglion cells. In the past decade or so, FM1-43 has been used extensively in goldfish ON-bipolar

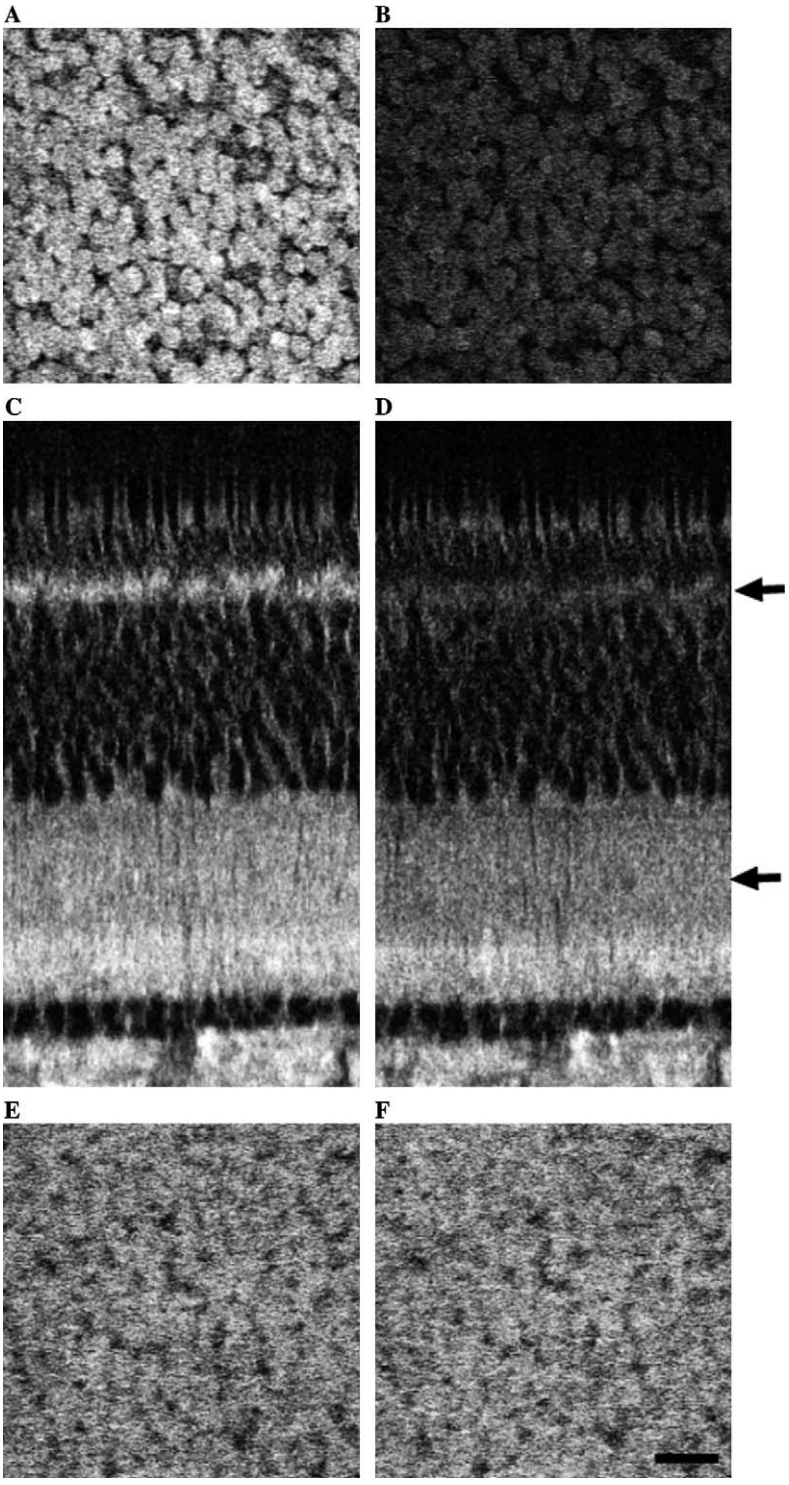

Fig. 3. Measuring light-regulated FM1-43 release from cone terminals (A and B) Tangential optical sections through the OPL of the lizard retinal flat-mount just after FM1-43 loading. (A)The cone synaptic terminals are labeled with FM1-43. (B) The cone synaptic terminals released their dye after 40 min in darkness. (C and D) Virtual slice views of the lizard retinal flat-mount. (C) Virtual slice view through the FM1-43-loaded retina shown in panel A. (D) After 40 min in darkness, OPL and OFF-sub-lamina within the IPL lost the dye (arrows). (E and F) Bright light suppresses release from the cone terminals in the OPL. (E) An optical section through the OPL just after FM1-43 dye loading, as in panel A. (F) The OPL of the same retina after exposure to bright light (10^7 photons/μm^2/s) for 20 min shows little change in fluorescence. Scale bar is 20 μm in all panels.

cells, but mostly to study subcellular events that govern vesicle release and recycling (for reviews see Guatimosim & von Gersdorff, 2002; von Gersdorff & Matthews, 1999). Almost all of these studies were done on acutely dissociated, individual bipolar cells, or on isolated terminals (Lagnado, Gomis, & Job, 1996; Rouze & Schwartz,

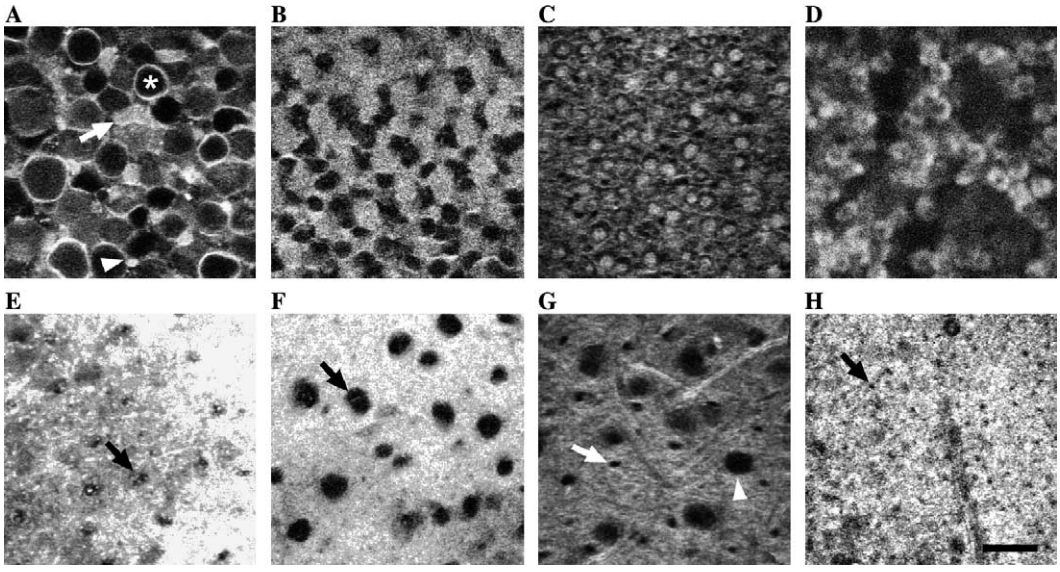

Fig. 4. FM1-43 labeling can be used in a wide variety of vertebrate retinas. (A–D) FM1-43-labeled photoreceptor terminals in the OPL in retinas from various vertebrate species. (A) Salamander OPL contains both cone (arrow) and rod (arrowhead) synaptic terminals. The large round structures (asterisk) are profiles of the photoreceptor cell bodies, whose synapses lie just below the focal plane. (B) Gecko retina has only rods and no cones. The OPL shows large rod synaptic terminals labeled with FM1-43. (C) Goldfish photoreceptor terminals are labeled with FM1-43. (D) Mouse rod synaptic terminals show FM1-43 labeling, which is brighter at the periphery than at the center. (E–H) FM1-43-labeled IPL in retinas from the same vertebrate species as above. Salamander (E), gecko (F), goldfish (G), and mouse (H) IPL are filled with small synaptic terminals of bipolar and amacrine cells and are brightly labeled with FM1-43. Muller cell processes appear dark throughout the IPL (arrows in E–H). In goldfish IPL, large ON bipolar cell presynaptic terminals also appeared dark (arrowhead in panel G). Scale bar is 20 μm in all panels except in panel D, where the scale bar is 5 μm.

1998; Zenisek, Steyer, & Almers, 2000). How light regulates dye release from bipolar cells terminals in the intact retina has not been examined.

To assess the activity of bipolar cell terminals in response to light, we examined dye uptake into the IPL. When the goldfish retina was exposed to room light in the presence of FM1-43 and normal extracellular Ca^{2+}, the Mb bipolar terminals became brightly labeled (Figs. 5A and C). Labeling was less intense in the center of the terminals, which is densely packed with mitochondria and therefore have a lower density of synaptic vesicles (Holt, Cooke, Neef, & Lagnado, 2004). When the goldfish retina was exposed to FM1-43 in darkness, the Mb bipolar terminals appeared dark (Figs. 5B and D). We quantified the FM1-43 labeling in the terminal loaded in the light and in darkness by examining the fluorescence intensity profile across the diameter of terminals (Fig. 5E). The intensity of the terminals loaded in the dark was much lower than that of the surrounding neuropil in the IPL, whereas terminals loaded in the light were much brighter than the background fluorescence in the IPL. A punctate labeling pattern was often seen around the perimeter of the terminals with both light and dark loading (arrows in Figs. 5C and D). These puncta may be loaded presynaptic terminals of amacrine cells that form axo-axonal synapses on the bipolar terminal (Kolb, 1997).

4. Conclusions

Using the activity-dependent synaptic vesicle dye FM1-43 together with 2-photon microscopy, we are beginning to see glimpses of how the retina as a whole responds to light. It is easiest to investigate release from giant presynaptic terminals, such as the cone terminals of anoles and the ON-bipolar cell terminals of goldfish. So far, our studies have been restricted to examining the effects of full-field, steady illumination, but it should be possible to examine how spatial patterns of light and dark influence synaptic release. This could help unravel the mystery of how horizontal cell feedback onto cone terminals mediates lateral inhibition in the outer retina. Stimuli with temporal contrast could also be used, but FM1-43 unloading from cone terminals is slow, limiting the temporal resolution of the release measurements. Nonetheless, our approach opens a new window for seeing how synapses in the intact retina function in response to visual stimuli.

Electroretinogram (ERG) recordings can monitor the activities of all types of neurons in the retina with excellent time resolution, but the method lacks resolution of single cells, and it is even difficult to separate the contribution of different cell types. Paired electrophysiological recordings from pre- and post-synaptic neurons are relatively invasive and it is difficult to expand to study more complex circuits involving many cells. In contrast,

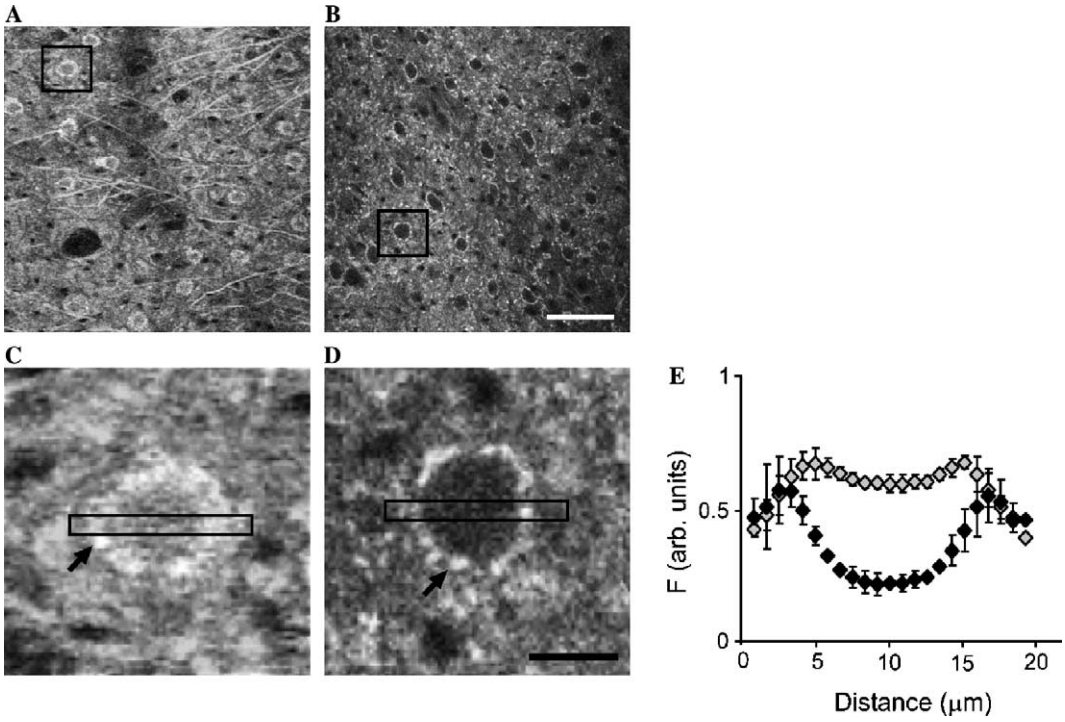

Fig. 5. Light-dependent FM1-43 labeling of goldfish ON-bipolar cell synapse. (A) Tangential optical section through the goldfish IPL after FM1-43 loading in room light for 45 min. The ON-bipolar cell terminals are labeled with FM1-43 throughout with the perimeter being brighter than the center. (B) Tangential optical section through the goldfish IPL after FM1-43 loading in darkness for 45 min. The ON bipolar cell terminals appear dark in the center. (C) Magnified view of a single terminal loaded with FM1-43 in the light as indicated in a box in panel A. (D) Magnified view of a single terminal loaded with FM1-43 in the dark as indicated in a box in panel B. Often we saw punctate structures labeled around the bipolar cell terminal (arrows in panels C and D). (E) FM1-43 fluorescence intensity profile across the diameter of bipolar cell synapses. Bipolar cell synaptic terminals loaded in the light show high intensity of labeling throughout (open diamonds), whereas those loaded in darkness show low fluorescence in the center (filled diamonds). Means ± SEM. Scale bar is 50 μm in (A) and (B), and 10 μm in (C) and (D).

FM1-43 can provide excellent spatial resolution of synaptic activity over large populations of cells. Here, we have shown changes in fluorescence before and 40–45 min after exposure to light or dark, but we have achieved better time resolution of release with more frequent 2-photon scanning (e.g., 1 or 2 min intervals; Choi et al., in press). It should be noted that higher time resolution means increased infrared laser exposure, raising concerns about possible 2-photon excitation of opsins (Euler, Detwiler, & Denk, 2002), potentially perturbing the very process we are trying to observe. Thus, there is a trade-off between achieving very high temporal resolution and preventing perturbation of photoreceptor activity. Nonetheless, the combination of activity-dependent dye and 2-photon microscopy enables recording from a large population of neurons with single cell resolution, hence it can certainly be a step forward in trying to understand retinal circuit interactions.

There are many other activity-dependent dyes that, when viewed with 2-photon miscopy, could be used to assess various aspects of retinal function. There are a wealth of small fluorescent molecule indicators of Ca^{2+} that could complement FM1-43 in reporting on presynaptic function. Other indicator dyes for Na^{+} and H^{+},could help describe and hopefully explain both pre- and post-synaptic events in both the outer and inner retina. Finally, a new generation of genetically-expressed GFP-based indicators are being developed for monitoring Ca^{2+} (e.g., cameleon; Fan et al., 1999), synaptic vesicle release (synaptophluorin; Bozza, McGann, Mombaerts, & Wachowiak, 2004), intracellular Cl^{-} (clomeleon; Kuner & Augustine, 2000), and membrane potential (FlaSh; Siegel & Isacoff, 1997). In theory, it should be possible to target these indicators to specific retinal cell types using cell-specific promoters. This would allow selective observation of retinal activity at different stages as visual information is processed.

Acknowledgments

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